GROWTH OF THE EDIBLE MUSHROOM *PLEUROTUS OSTREATUS* ON DIFFERENT CONCENTRATIONS OF DI (2-ETHYL HEXYL) PHTHALATE IN SOLID AND IN LIQUID MEDIA

GABRIELA CÓRDOBA-SOSA[2], ÁNGEL GONZÁLEZ-MÁRQUEZ[2], JOSÉ LUIS TORRES[3], MIRIAM AHUACTZIN -PEREZ[3], GERARDO DÍAZ-GODÍNEZ[1], RUBÉN DÍAZ[1]AND CARMEN SÁNCHEZ[1]*

[1]Laboratory of Biotechnology, Research Centre for Biological Sciences, Universidad Autónoma de Tlaxcala, Apdo. postal 129, Tlaxcala, México, CP 90000.

[2]Licenciatura en Biología, Universidad Autónoma de Tlaxcala, Ixtacuixtla, Tlaxcala, México, CP 90120.

[3]Facultad de Biología, Universidad Autónoma de Tlaxcala, Ixtacuixtla, Tlaxcala, México, CP 90120.

*sanher6@hotmail.com

ABSTRACT

Di (2-ethylhexyl) phthalate (DEHP) is one of the most widely used plasticizers, giving flexibility to the plastics. Specific growth rate (μ), maximum biomass (X_{max}), laccase and esterase activities, pH profiles and enzymatic kinetic parameters were evaluated in *Pleurotus ostreatus* grown in DEHP in flasks. Radial growth rate (u_r), laccase and esterase activities and mycelial biomass (X) of *P. ostreatus* grown in agar plates containing DEHP were also evaluated. Flasks of 125 ml and agar plates containing 0, 750, 1200 and 1500 mg of DEHP/l were used. All media were added with 10 g of glucose/l. Flasks containing 50 ml culture medium were inoculated and incubated at 25 °C for 16 days on a rotary shaker (120 rpm). Petri dishes were inoculated and incubated at 25 °C for 7 days. X_{max}, μ, and u_r were evaluated using the logistic and lineal equations, respectively. X was determined by dry weight method. Laccase and esterase activities were evaluated using 2, 6-dimethoxyphenol and *p*-nitrophenyl butyrate as substrates, respectively. The highest X_{max} was observed in media containing 1500 mg of DEHP/l and the esterase activity was much higher than the laccase activity at the beginning of the stationary phase in medium containing 1000 mg of DEHP/l in flasks. In agar plates, the laccase activity was higher than the esterase activity in all the media containing DEHP. These results suggest that there was no catabolite repression (glucose effect) and that DEHP was used as carbon and energy source by this fungus.

Keywords: di (2-ethylhexyl) phthalate, esterase, laccase, liquid medium, *Pleurotus ostreatus*, solid medium

INTRODUCTION

Di (2-ethylhexyl) phthalate (DEHP) belongs to the family of the phthalates or acid phthalic esters. More than 60 kinds of phthalates are produced nowadays [1]. These compounds are used each year as plasticizers in flexible polyvinyl chloride (PVC) products, resins, cellulosic, polyvinyl acetate and polyurethanes [2]. The annual worldwide production of phthalates exceeds 5 million tons [3]. DEHP is a high production volume chemical used in the manufacture of a wide variety of consumer food packaging, some children's products, and some polyvinyl chloride (PVC) medical devices.The European government banned the use of DEHP in toys and children's products that might be placed in the mouth (http://www.marchem.com/materials/plastisols/phthalatefree.html). It has been reported that phthalates are mutagenic, teratogenic and carcinogenic [4, 5]. In addition, phthalates are important environmental contaminants and are difficult to degrade easily. Elimination of DEHP by microorganisms is considered to be one of the major routes of environmental degradation. Hwang *et al*. [6] studied the degradation of 100 mg/l of butylbenzyl phthalate (BBP) by *P. ostreatus*. They found that the degradation of this compound was higher when the BBP was dispersed in an optimum liquid medium (yeast-malt extract-glucose) than in a minimal medium. They also reported that the esterases are more important than laccases in the degradation of this compound. On the other hand, *P. ostreatus* is the second most cultivated edible mushroom worldwide. This mushroom has a very important enzymatic machinery that is able to produce laccases and manganese peroxidases [7]. In this research, specific growth rate (μ), laccase and esterase activities, pH profiles and enzymatic kinetic parameters were evaluated in *P. ostreatus* grown in media containing 0, 750, 1200 and 1500 mg of DEHP/l in liquid media. Radial growth rate (μ), mycelial biomass and laccase and esterase activities were also determined in media added with 0, 750, 1200 and 1500 mg of DEHP/l in agar plates.

MATERIALS AND METHODS

Microorganism: *P. ostreatus* from the American Type Culture Collection (ATCC 3526) (Manassas, Virginia, U.S.A.) was used. The strain was grown on malt extract agar (MEA) at 25 °C and stored at 4 °C until used.

Culture media: Four liquid and four solid media were used. The liquid media had; 1) 50 ml of glucose-yeast extract medium (GY) + 0 mg of DEHP/l, 2) 50 ml of GY + 750 mg of DEHP/l, 3) 50 ml of GY + 1200 mg of DEHP/l and 4) 50 ml of GY + 1500 mg of DEHP/l. The solid media had the same liquid media composition plus 20 g of agar/l (this amount of agar was required as a solidifying agent). The YG medium had (in g/l): glucose, 10; yeast extract, 5; KH_2PO_4, 0.6; $MgSO_4.7H_2O$, 0.5; K_2HPO_4, 0.4; $CuSO_4.5H_2O$, 0.25; $FeSO_4.7H_2O$, 0.05; $MnSO_4$, 0.05 and $ZnSO_4.7H_2O$, 0.001. The pH was adjusted at 6.5 using either 0.1 M HCl or 0.1 M NaOH. To prepare solid media, the agar was added to the culture media and then the media were autoclaved. In solid and liquid media, the DEHP was added to the autoclaved media. The media containing the different concentrations of this compound were cool down at 50 °C approx. and sonicated for approximately 3 minutes using an ultrasonic processor (GEX 130) until the DEHP had been fully dispersed. The agar media were poured into Petri dishes [8]. We used these concentrations of DEHP, since in previous studies similar concentrations of this compound were used, and it was clearly observed the effect of dibutyl phthalate and DEHP on the growth of filamentous fungi [8].

Specific growth rate: Flasks of 125 ml containing 50 ml of the different culture media were inoculated with three mycelial plugs of 10 mm of diameter and incubated at 25 °C for 16 days on a rotary shaker at 120 rpm. The biomass (X) was obtained by filtration of the samples using filter paper (Whatman No. 4), and it was determined as difference of dry weight (g/l) [$X = X(t)$] using the Velhurst-Pearl or logistic equation (Equation 1). The X was measured daily until that the stationary phase of growth of the fungus started (16 days of incubation).

$$dX/dt=\mu[1-X/X_{max}]X \quad \text{or} \quad X[X_{max}/1+Ce^{-\mu t}] \quad \text{Equation 1}$$

Where, $X = X_0$ (the initial biomass value), $C = (X_{max} - X_0)/X_0$, μ is the maximal specific growth rate and X_{max} is the maximal (or equilibrium) biomass level achieved when $dX/dt = 0$ for $X > 0$. Thus, in logistic growth, the growth rate decreases as biomass increases. Evaluations of kinetic parameters of the logistic equation were carried out using a non-linear least square-fitting program (Solver; Excel, Microsoft) [9-11]. The value of μ was evaluated from the third to the 16 d of growth in order to avoid the variation adjustment problem.

Laccase and esterase activities in liquid medium: The supernatant obtained from the filtration of the samples corresponded to the enzymatic extract (EE). Laccase activity was determined in each EE by changes in the absorbance at 468 nm (using a Jenway 6405 UV/V is spectrophotometer), using 2, 6-dimethoxyphenol (DMP, SIGMA) as substrate. The assay mixture contained 900 µl of 2 mM DMP in 0.1 M acetate buffer pH 4.5 and 100 µl EE, which were incubated at 40 °C for 1 min. Esterases activity was determined by changes in the absorbance at 405 nm (using a Jenway 6405UV/Vis spectrophotometer), using *p*-nitrophenyl butyrate (*p*NPB) as substrate. The reaction mixture contained 10 µl of a *p*NPB solution [1.76 % (v/v) of *p*NPB in acetonitrile], 790 µl of 50 mM acetates buffer pH 7.0, 0.04% Tritón X-100 and 100 µl of the EE, which were incubated at 37 °C for 5 min [12, 13]. One enzymatic unit of laccase activity or esterase activity (U) is defined as the amount of enzyme which gives an increase of 1 unit of absorbance per min in the reaction mixture. The enzymatic activities were expressed in U/l of EE.

Enzymatic kinetic parameters: The enzymatic kinetic parameters were evaluated in those cultures grown in liquid medium. Yield of laccases per unit of biomass produced ($Y_{E/X}$) was estimated as the relation between maximal enzymatic activity (E_{max}) and X_{max} (see specific growth rate in the methodology section). Enzymatic productivity (P = U/l h) was evaluated using the time of E_{max}. The specific rate of enzymatic production was calculated by the equation; $qP = (\mu)(Y_{E/X})$ [10, 11, 14]

Radial growth rate, biomass and enzymatic assays in solid medium: Petri dishes were inoculated in the center with a plug of 4 mm of diameter and incubated at 25 °C for 7 days. The radius of the mycelial growth was measured daily from

2 to 7 days of incubation using a vernier (digital mitutoyo). The radial growth rate (u_r) was calculated as the slope of the radius versus time plots, analyzed by lineal regression [8, 15]. The *X* was evaluated in 7 days old colonies. The mycelium was separated from the culture medium using a boiling water bath and then placed in a pre-weighed watch glass. It was weighed, and then oven-dried at 60 °C for 24 h, then weighed again [8]. In solid media, the laccase and esterase activities were evaluated in 7 d old grown colonies. The colonies grown on agar were flooded with distilled water and left at room temperature for 24 h, after which the water was removed from the Petri dish and then centrifuged. The centrifuged water was considered the EE. Esterase and lacasse activities were determined as indicated above (see laccase and esterase activities in liquid medium in the methodology section). One enzymatic unit of esterase activity or laccase activity (U) is defined as the amount of enzyme which gives an increase of 1 unit of absorbance per min in the reaction mixture. The enzymatic activities were expressed in U/l of EE.

Statistical analysis: All the experiments were carried out by triplicated. Data were evaluated using one-way ANOVA and Tukey post-test using The Graph Pad Prism® program.

RESULTS

In liquid medium, the highest μ was obtained in medium containing 750 mg of DEHP/l, followed by the media containing 1200 mg of DEHP/l, 1500 mg of DEHP/l and the medium lacking DEHP (Equation 1, Fig. 1, Table 1). The highest X_{max} was obtained in the culture media with addition of 1500 and 1200 mg of DEHP/l, and the lowest X_{max} was showed in the medium containing 750 mg of DEHP/l (equation 1, Table 1). In solid medium, the highest u_r was observed in the medium lacking DEHP followed by the media containing 1500, 750 and 1200 mg of DEHP/l. The highest and lowest *X* was obtained in media with addition of 1500 mg and without added DEHP, respectively (Table 1).

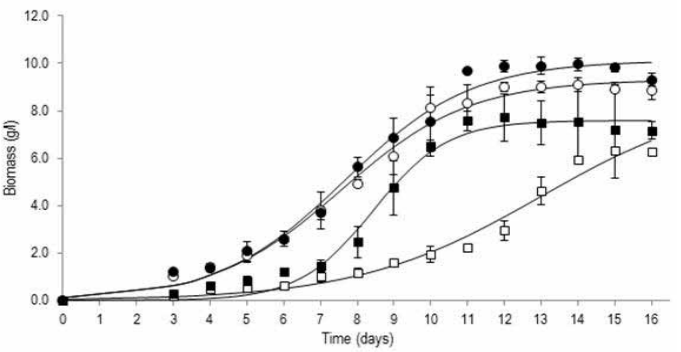

Figure 1. Specific growth rate of *P. ostreatus* grown in 0 (□), 750 (■), 1200 (○) and 1500 (●) mg of DEHP/l in liquid medium. The experimental data of μ were adjusted using the equation 1

Table 1. Radial and specific growth rates, X_{max} and *X* of *P. ostreatus* grown in different concentrations of DEHP.

Parameter	Culture system	Culture media DEHP (mg/l)			
		0	750	1200	1500
μ (1/h)	Liquid	0.016^{b} (0.001)	0.041^{a} (0.007)	0.024^{b} (0.003)	0.024^{b} (0.003)
X_{max} (g/l)	Liquid	8.78^{d} (0.27)	7.59^{c}(0.64)	9.31^{abc} (0.017)	10.12^{ab} (0.17)
u_r (mm/d)	Solid	0.49^{a}(0.009)	0.41^{c}(0.003)	0.40^{d}(0.017)	0.42^{b}(0.001)
X (mg/cm^2)	Solid	0.116^{d}(0.008)	0.125^{c}(0.010)	0.13^{b}(0.017)	0.14^{a}(0.006)

Means with the same letter within a row are not significantly different. Numbers in parenthesis correspond to standard deviation of three separate experiments.

In liquid media, the highest activity of laccase was showed at the end of the exponential phase of growth (13 d of fermentation) in the media containing 1500 mg of DEHP/l (Fig. 2a). The highest activity of esterase was observed at the beginning of stationary phase (15 day of fermentation) in media containing 1500 mg of DEHP/l (Fig. 2b). In general, the lowest activities of laccase and esterase were observed in the medium lacking DEHP and in the medium containing 750 mg of DEHP/l (Figs. 2a, b). In general, the activity of esterase was observed in all the media containing DEHP, however, the activity of

laccase was only observed in media containing 1500 and 1200 mg of DEHP/l (Figs. 2a, b). In general, the medium lacking DEHP showed the lowest activities of laccase and esterase (Figs. 2a, b). The production of esterase at the beginning of the stationary phase of growth was similar in the media containing 0, 750, 1200 g of DEHP/l (Fig. 2a).

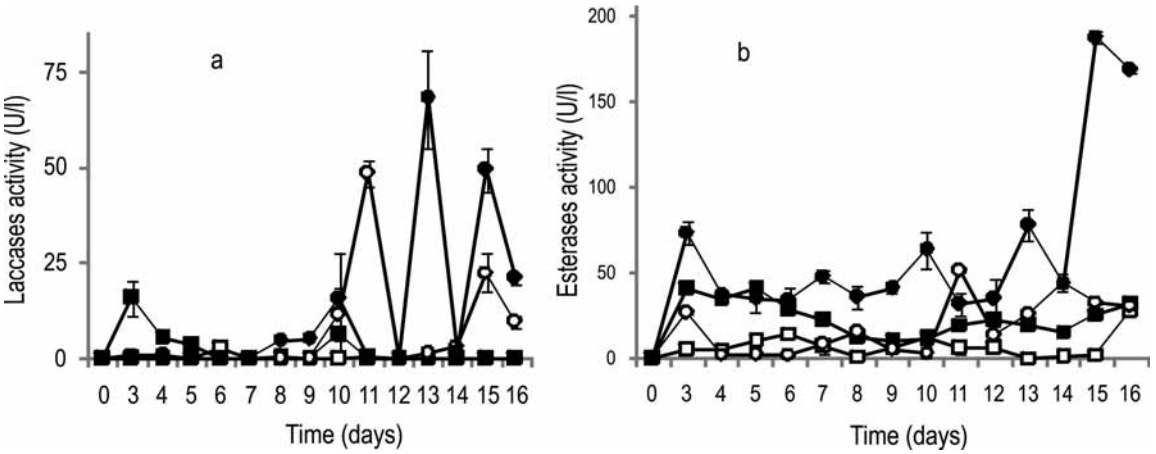

Figure 2. Laccases (a) and esterases (b) activities of *P. ostreatus* grown in 0 (□), 750 (■), 1200 (○) and 1500 (●) mg of DEHP/l in liquid medium

The highest laccase and esterase kinetic parameters were observed in 1500 mg of DEHP/l, followed by the rest of the culture media DEHP/l (Tables 2, 3). From all the culture media, the medium containing 1500 mg of DEHP/l had the lowest pH value (during the 3 d of growth) (5.4 approx.).

Table 2. Laccase kinetic parameters of *P. ostreatus* grown in liquid medium in different concentrations of DEHP.

Parameter	**Culture media DEHP (mg/l)**			
	0	**750**	**1200**	**1500**
E_{max} (U/l)	2.80^{c}(0.35)	15.80^{c}(0.95)	48.347^{b}(3.522)	67.845^{a}(12.89)
$Y_{E/X}$ (U/g)	0.3184^{b}(0.0317)	2.0034^{b}(0.0519)	5.1807^{a}(0.3843)	6.6898^{a}(1.2993)
qP(U/g/h)	0.0053^{d}(0.0002)	0.0840^{c}(0.0150)	0.1242^{b}(0.0051)	0.1603^{a}(0.0161)
P (U/l/h)	0.0195^{b}(0.0024)	0.02201^{a}(0.0132)	0.1831^{a}(0.0133)	0.2175^{a}(0.0413)

Means with the same letter within a row are not significantly different. Numbers in parenthesis correspond to standard deviation of three separate experiments.

Table 3. Esterase kinetic parameters of *P. ostreatus* grown in liquid medium in different concentrations of DEHP.

Parameter	**Culture media DEHP (mg/l)**			
	0	**750**	**1200**	**1500**
E_{max} (U/l)	27.8447^{d}(0.6219)	41.2932^{c}(3.3415)	51.4021^{b}(0.6411)	188.4142^{a}(3.3126)
$Y_{E/X}$ (U/g)	3.1687^{c}(0.1565)	5.2432^{b}(0.6895)	5.5078^{b}(0.0770)	18.5692^{a}(0.4348)
qP(U/g/h)	0.0529^{c}(0.0055)	0.2228^{b}(0.0664)	0.1326^{cb}(0.0129)	0.4506^{a}(0.0385)
P (U/l/h)	0.0725^{d}(0.0016)	0.3441^{c}(0.0278)	0.1947^{b}(0.0024)	0.5234^{a}(0.0092)

Means with the same letter within a row are not significantly different. Numbers in parenthesis correspond to standard deviation of three separate experiments.

The media containing 1200 and 1500 g of DEHP /l showed a similar pH profile. The pH profiles of the medium added with 750 mg of DEHP/l and medium lacking DEHP were similar (Fig. 3).

The pH of the media containing 1200 and 1500 mg of DEHP/ l showed higher pH than the rest of the culture media at the end of the fermentation. In solid medium, the laccases activity was much higher than the esterase activity in all the culture media. The highest laccases activity (Fig. 4a) and esterase activity (Fig. 4b) was observed in 1500 mg of DEHP/l, followed by that activity shown in the media containing 750 mg of DEHP/l, 1200 mg of DEHP/l and without added DEHP. The lowest activity of both enzymes was observed in media lacking DEHP.

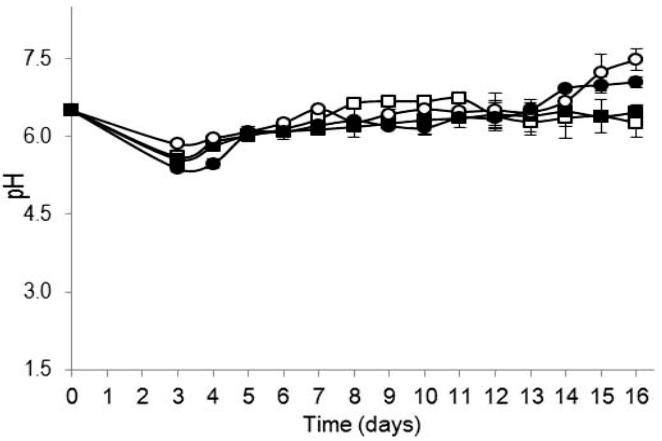

Figure 3. Profile of pH of *P. ostreatus* grown in 0 (□), 750 (■), 1200 (○) and 1500 (●) mg of DEHP/l in liquid medium

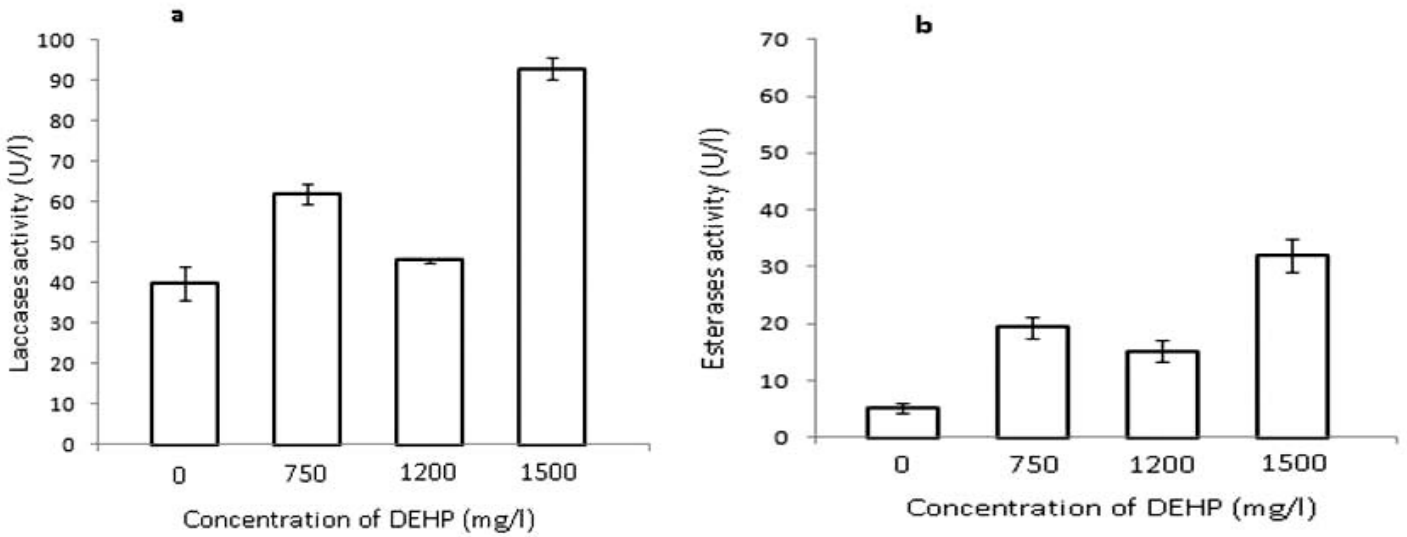

Fig. 4. Laccases (a) and esterases (b) activities of a 7 d old colony of *P. ostreatus* grown in solid medium in different concentrations of DEHP

DISCUSSION

In liquid media, the μ and the highest X_{max} were calculated using the logistic equation [14] (Eq. 1), fitting to an exponential growth model. In logistic growth, the growth rate decreases as biomass increases. These results showed that the DEHP is used by this fungus to grow, since the media containing the highest amount of DEHP showed the lowest μ and the highest X_{max}(Table 1). It is known that, from 100% of carbon source added to a culture media, 50% is used by microorganisms for biomass production and 50% for structure formation. In this study 10 g glucose/l were added to all the cultures (see materials and methods), since a diauxic growth might occurs in some microorganism that grow on complex compounds [6]. The X_{max} produced in medium lacking DEHP was approx. 5 g/l (amount that corresponded to 50% of 10 g/l of glucose that was added to all the media (Table 1). These results showed that the DEHP was used as carbon and energy sources, since the biomass production was enhanced as the concentration of DEHP increased (Table 1). On the other hand, in solid medium, the highest X was obtained in the media containing DEHP, however, the highest u_r was observed in the medium lacking DEHP (Table 1). Suarez-Segundo *et al.* [8] reported that the growth (μ) of some strains of filamentous fungi on a minimal medium lacking DEHP could be due to the use of certain amount of nutrients that unavoidably remain in the inoculum and/or the use of nutrients produced by cellular lysis of the hyphae from the inoculum. The pH increased during the fermentation in the media containing 1200 and 1500 mg of DEHP/l. This could be due to the degradation of this

compound, releasing basic breakdown products of DEHP (http://umbdd.ethz.ch/index.html). The degradation of DEHP has been studied in liquid medium in shake flasks or laboratory scale fermenters using pure or mixed cultures or acclimatized activated sludge. In all cases, DEHP was observed to be readily degradable [16]. Chatterjee and Dutta [17] reported that *Gordonia* sp. and *Arthrobacter* sp. utilized butylbenzyl phthalate (BBP) individually as the sole source of carbon and energy. Hwang *et al*. [6] studied the addition of 100 mg/l of BBP to yeast-malt extract-glucose culture medium and reported that the esterases activity was induced by BBP itself and that these enzymes were more important than the laccases in the BBP degradation by *P. ostreatus* in liquid medium. Similarly, we found that *P. ostreatus* had higher activity of esterase than activity of laccase in liquid medium containing different concentrations of DEHP. It has been reported that the 16-hydroxihexadecanoic acid, a cutin monomer, was able to induce the esterase in the saprophytic fungus *Glomerella cingulate* [18]. Van der Vlught-Bergman *et al*. [19] studied the growth of the white rot fungus *Phlebia tremellosa*, in liquid medium containing benzylbutyl phtalate and diethyl phtalate in concentration of 30% and 80%, respectively, and found that the fungus increased the laccase production after 9 days of growth. On the other hand, the maximal enzymatic activity, enzymatic productivity and rate of the enzymatic production depend on the amount of phthalate added to the liquid medium (Tables 2 and 3). The kinetic parameters were higher in the medium containing 1500 mg of DEHP/l than in the rest of the media. It shows that high concentrations of DEHP increased the yield of enzyme and enhanced the metabolism of the strain. In solid medium, the laccases activity was higher than the activity of esterase in all the media (Figs. 4a, b). This might be due to the fact that laccases are very important in the mycelial invasion of solid substrates. These results show that the type of enzyme produced during the DEHP degradation depends, at least in part, on the culture system and on the DEHP concentration. The production of laccase and esterase, and the results of the growth of *P. ostreatus* in the medium containing 1500 mg of DEHP/l suggest that there was no catabolite repression (glucose effect) and that DEHP was used as carbon and energy source.

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REFERENCES

[1] Lu Y *et al*. (2009). Biodegradation of dimethyl phthalate, diethyl phthalate and di-n-butyl phthalate by *Rhodococcus* sp. L4 isolated from activated sludge. *J. Hazard. Mater*. 168: 938-943.

[2] Vats S *et al*. (2013). Phthalates- A priority pollulant. *Int. J. Adv. Biol. Res.* 3: 1-8.

[3] Mackintosh C *et al*. (2006). Sorption of phthalate esters and PCBs in a marine ecosystem. *Environ. Sci. Technol*. 40: 3481-3488.

[4] Manikkam M *et al*. (2013). Plastics derived endocrine disruptors (BPA, DEHP and DBP) induce epigenetic transgenerational inheritance of obesity, reproductive disease and sperm epimutations. *PLoS ONE* 8:e55387. DOI:10.1371/journal.

[5] Dobrzyñska MM *et al*. (2012). Two generation reproductive and developmental toxicity following subchronic exposure of pubescent male mice to di(2-ethylhexyl)phthalate. *Ann. Agr. Environ. Med*. 19: 31-37. pone. 0055387. 2013.

[6] Hwang SS *et al*. (2012). Changes in the activities of enzymes involved in the degradation of butylbenzyl phthalate by *Pleurotus ostreatus*. *Microbiol. Biotechnol.* 22: 239-243.

[7] Sánchez C. (2009). Lignocellulosic residues: biodegradation and bioconversion by fungi. *Biotechnol. Adv*. 27: 185-194.

[8] Suárez-Segundo JL *et al*. (2013). Growth of colonies and hyphal ultrastructure of filamentous fungi grown on dibutyl phthalate and di (2-etilhexil) phthalate. *Rev. Mex. Ing. Quim.* 12: 499-504.

[9] Viniegra-González G *et al*. (2003). Advantages of fungal enzyme production in solid state over liquid fermentation systems. *Biochem. Eng. J.* 13: 157-167.

[10] Díaz R *et al*. (2011). Characterization of the growth and laccase activity of strains of *Pleurotus ostreatus* in submerged fermentation. *Bio Resources*. 6: 282-290.

[11] Díaz R *et al*. (2013). Influence of initial pH of the growing medium on the activity, production and expression profiles of laccases produced by *Pleurotus ostreatus* in submerged fermentation. *Electron. J. Biotechnol*. 16: 1-13.

[12] Alves-Macedo G and Fontes-Pio T. (2005). A rapid screening method for cutinase producing microorganisms. *Braz J Microbiol.* 36: 388-394.

[13] Kasturi A *et al*. (2009). Production, characterization and applications of microbial cutinases. *Process. Biochem.* 44: 127-134.

[14] Viniegra-González G *et al*. (2003). Advantages of fungal enzyme production in solid state over liquid fermentation systems. *Biochem. Eng. J*. 13: 157-167.

[15] Sánchez C and Viniegra-Gonzaléz G.. (1996). Detection of highly productive strains of *Pleurotus ostreatus* by their tolerance to 2-deoxy-D-glucose in starch-based media. *Mycol. Res.*100: 455-461.

[16] Juneson C *et al*. (2001). Biodegradation of bis (2-ethylhexyl) phthalate in a soil slurry-sequencing batch reactor. *Process. Biochem.* 37: 305-313.

[17] Chatterjee S, Dutta TK. (2008). Complete degradation of butyl benzyl phthalate by a defined bacterial consortium: role of individual isolates in the assimilation pathway. *Chemosphere*. 70: 933-41.

[18] Bakar FDA *et al*. (2005). Induction and expression of cutinase activity during saprophytic growth of the fungal plant pathogen, *Glomerella cingulata*. *Asia. Pac. J. Mol. Biol. Biotechnol.* 13: 63-69.

[19] Van der Vlugt-Bergmans CJ *et al*. (1997). Cloning and expression of the cutinase a gene of *Botrytis cinerea*. *Mol. Plant. Microb. Interact.* 10: 21-29.